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CBRN Defence Centre
Platforms Sciences Laboratory

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ABSTRACT

Melioidosis is a potentially lethal infection endemic in Northern Australia and Southeast Asia. The causative bacterium, *Burkholderia pseudomallei*, is resistant to many of the commonly used antibacterial agents. Therefore, successful treatment of the disease is dependent on rapid and accurate diagnosis. The current diagnostic techniques are time consuming and lacking in specificity. We have developed a PCR procedure using the R.A.P.I.D™ and real time monitoring of product formation. The reaction is significantly faster than conventional PCR and allows the simultaneous evaluation of specificity using melting curve analysis.

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506 Lorimer St
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Telephone: (03) 9626 7000
Fax: (03) 9626 7999

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Polymerase Chain Reaction for the Rapid Detection of *Burkholderia pseudomallei*

Executive Summary

The aim of this project was to develop a polymerase chain reaction (PCR) procedure to detect the bacterium, *Burkholderia pseudomallei*. The organism causes the disease melioidosis, which is of concern to the Australian Defence Force for a number of reasons. The infection is potentially lethal, it is endemic in possible deployment regions, passive immunological protection is not available, and since the bacterium is resistant to many of the commonly used antibacterial agents effective treatment is dependent on a correct diagnosis.

PCR synthesises DNA using a specified template through repetitive cycles of temperature changes. PCR was chosen as a detection method because it is rapid, specific and real-time monitoring is possible using fluorescent dyes or probes such as SYBR Green I. In addition, due to advances in the technology automation of the assay is feasible in the near future.

In the optimisation of the assay, a number of parameters were adjusted to determine the optimal concentrations of reagents without compromising either the speed or specificity of the reaction. The duration of the assay was 40 min, a significant reduction from the 2.5 hours required for the conventional PCR method. The specificity of the assay in detecting *B. pseudomallei* DNA was demonstrated using template DNA extracted from several bacterial strains.

Authors

Susan Shahin
Chemical, Biological, Radiological, Nuclear
Defence Centre

Susan Shahin, PhD, joined the CBRN Defence Centre in 1998. Prior to DSTO, Susan worked at the University of Melbourne investigating the regulatory mechanisms of protein folding in yeast. Her work in DSTO includes the damaging effects of Sulphur mustard on mammalian cell DNA and development of rapid PCR techniques for the detection of biological warfare agents.

Matthias Dorsch
Chemical, Biological, Radiological, Nuclear
Defence Centre

Matthias Dorsch graduated with a diploma from Christian Albrechts University, Kiel, Germany, in 1987 and was awarded a Ph. D. in 1990 from the same University. The topic of the thesis was the phylogeny of Gram-positive eubacteria. He conducted postdoctoral research at The University of Queensland, The University of New South Wales and Macquarie University. During this time he specialised in detection and identification of bacterial and protozoan pathogens and indicator organisms. He joined the Platforms Sciences Laboratory in 2001. The focus of his research is the development of rapid detection and identification methods for biological warfare agents.

Contents

1. Introduction

Burkholderia pseudomallei is the causative bacterial agent of the disease meloidosis, which is endemic in Northern Australia and Southeast Asia. For a review on the current knowledge of the pathogenesis of the bacteria, mode of transmission and treatment options see Shahin (2000). Since the bacterium is resistant to a number of the commonly used antibiotics, effective treatment is dependent on rapid and accurate diagnosis. The most reliable identification technique involves the culturing of clinical samples which is time consuming, often taking days. Time is an important consideration, especially with respect to the septicemic form of the disease, which can be fatal within 2 days. Immunological assays based on the presence of specific antibodies are much faster but are susceptible to false positives, particularly in endemic areas.

The aim of this project was to develop a polymerase chain reaction (PCR) procedure to detect the bacteria in real time. There are a number of PCR techniques described in the literature (Haase *et al*, 1998; Bauernfeind *et al*, 1998; Zysk *et al*, 2000) using a range of primers and cycling conditions. However, these assays all use conventional thermal cyclers, which when coupled with the mandatory analysis of products on an agarose gel requires significant time. In addition, to increase specificity, researchers have added steps such as a secondary PCR or labelled hybridization probes (Dharakul et al., 1996; Sura et al., 1997) which tend to considerably delay the final result. The R.A.P.I.D™ thermal cycler was chosen since this technology allows real-time monitoring using fluorescent probes or dyes and is significantly faster than conventional PCR machines. In addition, specificity can be assessed using melting curve analysis.

2. Materials and Methods

2.1 Bacterial Strains

The following strains of bacteria were obtained from the American Type Culture Collection (ATCC) of Microorganisms: *Burkholderia cepacia* (ATCC 25416); *Staphlococcus aureus* (ATCC 9144); *Shigella sonnei* (ATCC 9290) and *Erwinia chrysanthemi*. The *Burkholderia pseudomallei* strain 08 was kindly provided by Professor Ifor Beacham (Griffith University, Queensland). *Escherichia coli* DH5α strain was obtained from Promega.

2.2 Preparation of genomic DNA

A single colony of *B. pseudomallei* or *E. coli* was inoculated into 10 ml of LB broth and incubated at 37°C for 24-48 hours with shaking. Genomic DNA was extracted and purified with the Qiagen Tissue Kit (QIAGEN GmbH, Germany) according to the manufacturer's instructions with the following modifications. Cells from 4 ml of culture fluid were pelleted and lysed overnight with proteinase K and the DNA eluted from the column with 100 µl H_2O at 70°C for 2 min. The DNA concentration was determined by measuring absorbance at 260 nm.

Lyophilised stocks of *B. cepacia*, *S. sonnei* and *E. chrysanthemi* were reconstituted in 10 ml of nutrient (Oxoid) broth and incubated at 37°C (*S. sonnei*) or 28°C (*B. cepacia*, *E. chrysanthemi*). *S. aureus* was grown in LB broth at 37°C. Colonies were cultured on either LB or nutrient agar plates and 10 ml of broth inoculated with a single colony for DNA extraction. Genomic DNA was prepared using the QIAGEN kit according to the manufacturer's instruction. Briefly, cells from 4 ml of culture were resuspended in 2.8 mg/ml lysozyme (20 mM Tris-HCL, pH 8.0; 2.0 mM EDTA; 1.2% triton). Lysis was performed at 37°C for 30 min and following the addition of proteinase K (2.0 mg/ml) incubated at 56°C for a further 30 min. Concentration of DNA was determined by absorbance at 260 nm.

Samples of *Y. pestis* and *B. anthracis* cells were obtained from vaccines containing heat killed cells or attenuated (Sterne strain 34F2) cells respectively. The *Y. pestis* vaccine was purchased from CSL Limited (Australia) and the Colorado Serum Company (United States) was the source of the *B. anthracis* vaccine. Genomic DNA from *Y. pestis* and *B. anthracis* was prepared as follows: a sample of vaccine was lysed with 5 cycles of freezing on dry ice for 2 min and boiling for 2 min followed by phenol/chloroform extraction. Genomic DNA from *C. burnetii* was kindly provided by Dr John Bates (Queensland Health Scientific Services, Brisbane).

2.3 PCR amplification

2.3.1 Primer Design

Primers were designed using DNA sequences from the GenBank non-vertebrates sequence database. Programs used for comparative sequence analysis were 'eclustalw' and 'fasta'. Both programs are available through ANGIS (Australian National Genomic Information Service). Primer sequences were determined from a multiple sequence alignment created with the program eclustalw. The alignment included the complete 23S rDNA sequences from *B. pseudomallei*, *B. mallei* and a number of phylogenetically closely related species. Species and GenBank sequence accession numbers were: *Burkholderia pseudomallei*, Y17184; *Burkholderia mallei*, Y17183; *Burkholderia cepacia*, X16368; *Burkholderia gladioli*, Y17182; *Burkholderia multivorans*, Y18704; *Burkholderia vietnamensis*, Y18705; *Ralstonia pickettii*, AF012421 and *Ralstonia solanacearum*, AF012419. The target sequences of the primers were then subjected to comparative analysis against the entire GenBank database using the fasta program to confirm specificity. According to comparative analysis the forward primer is complementary to the 23S rDNA of all species listed above whereas the reverse primer exhibits complete homology only to the 23S rDNA of *B. mallei* and *B. pseudomallei* (Figure 1). The corresponding target region in the 23S rDNA of the other species shows a sufficient number of sequence differences to enable selective amplification of *B. mallei* and *B. pseudomallei* 23S rDNA.

The primers BpsF (5' - GGT GCA AGC TCT TGA TTG AAG TCC – 3'; Tm 57°C) and BpsR (5' - ATC AAG TCC CCG GGG AAG – 3'; Tm 52°C) amplified a 931 bp fragment from position 1850 bp to 2781 bp of the 23S rRNA gene.

The universal primers designed to amplify a 1500 bp fragment of the 16S rRNA gene were 11-29F (5' – GAG TTT GAT CCT GGC TCA G – 3'; Tm 51°C) and 1492-1512R (5'–ACG GCT ACC TTG TTA CGA CTT – 3'; Tm 52°C) (Dorsch and Stackebrandt, 1992).

Primers were synthesised by Gene Works (Thebarton, SA).

	1850	2021	2039	2110	2120
Ralstonia solanacearum	**GGTGCAAGCTCTTGATTGAAGTCC**	C	N	T	GAC
Ralstonia pickettii	**GGTGCAAGCTCTTGATTGAAGTCC**	C	N	T	GAC
Pseudomonas cepacia	**GGTGCAAGCTCTTGATTGAAGTCC**	A	A	A	AAC
Burkholderia gladioli	**GGTGCAAGCTCTTGATTGAAGTCC**	C	A	A	AAC
Burkholderia mallei	**GGTGCAAGCTCTTGATTGAAGTCC**	A	A	A	AA**T**
Burkholderia pseudomallei	**GGTGCAAGCTCTTGATTGAAGTCC**	A	A	A	AA**C**
Burkholderia multivorans	**GGTGCAAGCTCTTGATTGAAGTCC**	A	A	A	AAC
Burkholderia vietnamiensis	**GGTGCAAGCTCTTGATTGAAGTCC**	A	A	A	AAC

	2142	2184	2191	2197	2043	2150	2164	2177	2190	2196
Ralstonia solanacearum	A	C	A	G	T	T	T	T	C	ACCTAA
Ralstonia pickettii	T	C	A	G	C	T	T	T	C	ACCTAA
Pseudomonas cepacia	T	C	-	G	C	C	A	A	A	GCTGAT
Burkholderia gladioli	T	C	-	G	C	T	A	A	A	GCTGAT
Burkholderia mallei	T	C	-	G	C	C	A	A	A	GCTGAT
Burkholderia pseudomallei	T	C	-	G	C	C	A	A	A	GCTGAT
Burkholderia multivorans	T	T	-	A	C	G	A	A	A	GCTGAT
Burkholderia vietnamiensis	T	C	-	G	C	C	A	A	A	GCTGAT

	2214	2364	2482	2535	2613	2620	2659	2662	2673	2679	2687
Ralstonia solanacearum	A	A	G	T	ATC	C	T	G	T	CT	-A
Ralstonia pickettii	A	A	G	T	ATC	C	T	G	T	CT	-A
Pseudomonas cepacia	T	T	G	T	TAT	A	A	T	G	CA	TG
Burkholderia gladioli	T	T	C	G	TAT	A	A	T	G	CA	TG
Burkholderia mallei	T	T	G	T	AGT	A	A	T	G	GA	TC
Burkholderia pseudomallei	T	T	G	T	AGT	A	A	T	G	GA	TC
Burkholderia multivorans	T	T	G	T	TAT	A	A	T	G	GA	TC
Burkholderia vietnamiensis	T	T	G	T	TAT	A	A	T	G	CA	TG

	2696	2709	2731	2749	2754	2763	2781
Ralstonia solanacearum	A	A	-	C	G	G**ATTCCCTGGAGACTTGAT**	
Ralstonia pickettii	A	A	-	T	G	G**ATTCCCTGGAGACTTGAT**	
Pseudomonas cepacia	C	T	G	C	T	A**TATCCCTGGGGACTAGAT**	
Burkholderia gladioli	C	T	C	C	T	A**TATCCCTGGAGGCTTGAC**	
Burkholderia mallei	C	T	G	C	T	A**CTTCCCCGGGGACTTGAT**	
Burkholderia pseudomallei	C	T	G	C	T	A**CTTCCCCGGGGACTTGAT**	
Burkholderia multivorans	C	T	G	C	T	A**TATCCCTGGAGGCTTGAC**	
Burkholderia vietnamiensis	C	T	G	C	T	A**TATCCCTGGGGACTAGAT**	

Figure 1: 23S alignment of B.pseudomallei and related species in the region flanked by the primers. The primers are highlighted in bold and underlined. The single nucleotide difference between B. pseudomallei and B. mallei described by Bauernfeind et al (1998) is also highlighted (position 2122 below).

2.3.2 Assay Conditions

PCR amplification reactions were performed in a total volume of 20 µl using the FastStart DNA polymerase (Roche) in the R.A.P.I.D.™ (Idaho Technologies Inc.) with SYBR Green I as the fluorophore. To compare the products, melting curve analysis was carried out routinely. The melting temperature (Tm) refers to the temperature at which 50% of the double stranded DNA dissociates.

The amount of template used was 15 ng of genomic DNA in reactions containing 4.0 mM $MgCl_2$ and 1.0 µM of each primer. The DNA polymerase was activated for 5 min at 95°C and template DNA amplified for 40 cycles of denaturation at 95°C for 0 sec, annealing at 52°C for 10 sec and elongation at 72°C for 60 sec. The time of 0 sec instructs the cycler to reach the temperature specified and immediately continue to the next step. A negative control consisting of all of the assay reagents except template DNA was included in all experiments.

3. Results

3.1 Optimisation of the PCR for the detection of *B.pseudomallei*

The FastStart DNA Master SYBR Green I (Roche) contains FastStart Taq DNA polymerase with heat labile blocking groups. The enzyme becomes activated when these groups are removed at high temperatures. To determine the minimum time required to activate the enzyme without adversely affecting product formation, the effect of varying the initial time of denaturation and the denaturation time in the cycling phase was examined (Figure 2). Heating the samples for 2 min at 94°C did not completely activate the enzyme as indicated by the high number of cycles (greater than 30) required before any significant product was detected. Similarly, even though increasing the denaturation time to 10 min allowed detection of product between 15 and 20 cycles, the PCR profile differed little from a denaturation time of 5 min. Therefore, an initial denaturation of 5 min followed by denaturation for 0 sec in the cycling phase and primer annealing for 10 sec which yielded the best PCR profile (Figure 2) was chosen. Similarly, an annealing temperature of 55°C generated the best profile compared to annealing temperatures of 53°C and 57°C (Figure 3).

Using the above parameters, the optimal amount of *B. pseudomallei* DNA template was determined (Figure 4). There was little difference in the amplification profile between 15 ng and 30 ng and subsequent reactions were therefore carried out using 15 ng of template. The optimal concentration of the co-factor Mg^{2+} required was 4.0 mM (Figure 5). Lower concentrations of Mg^{2+} were ineffective and the reaction was inhibited by the higher concentration of 5.0 mM (Figure 5). Similarly, the effect of primer concentrations was tested and concentrations of 1.0 µM were chosen based on the generation of detectable product with fewer cycles (Figure 6).

In summary, the optimal concentrations of the PCR components for the detection of *B. pseudomallei* DNA are: 15 ng of genomic DNA template; 4.0 mM $MgCl_2$ and a concentration of 1.0 µM for each of the primers BpsF and BpsR in a total volume of 20 µl. The assay

conditions using the R.A.P.I.D.™ thermal cycler and the FastStart DNA Master SYBR Green I are: an initial denaturation of 5 min at 95°C to activate the polymerase followed by 30 cycles of denaturation (95°C) for 0 sec, annealing at 55°C for 10 sec and elongation at 72°C for 40 sec.

3.2 Specificity of the *B. pseudomallei* PCR

To determine the specificity of the Bps primers using the cycling conditions described above, the genomic DNA from a number of bacterial strains was used as a template. The genomic DNA was extracted as described in the Methods and, as a control, a fragment of the 16S rRNA gene was amplified using the universal primers 11-29F and 1492-1512R. All of the genomic DNA samples were effective as templates for the amplification of the 16S rRNA gene fragment (Figures 7 and 9). Conversely, when used as templates with the Bps primers and amplified using the *B. pseudomallei* PCR cycling parameters, only *B. pseudomallei* resulted in significant product formation (Figures 8 and 10). A product was formed after 30 cycles with *Y. pestis* and *C. burnetii* (Figure 8) and with *E. coli* and *E. chrysanthemi* (Figure 10) genomic DNA. Melting curve analysis suggests that the fragments amplified using *C. burnetii* DNA (Figure 11) and both *E. coli* and *E. chrysanthemi* (Figure 12) were due to primer – dimer complex formation. However, the *Y. pestis* product displayed a similar Tm to the *B. pseudomallei* product (Figure 11).

4. Discussion

We have designed a PCR assay to detect *B. pseudomallei* DNA rapidly using the R.A.P.I.D™ thermocycler and the fluorophore SYBR Green I. Ribosomal DNA was chosen as the target because rDNA operons in bacteria are usually multi-copy genes that provide increased sensitivity for PCR assays as compared to single copy genes. If required, the assay sensitivity can be further increased by introducing a reverse transcriptase PCR to amplify the transcripts of the rDNA genes, the rRNAs, which can comprise up to 45,000 copies per cell (Fegatela et al., 1998). The 16S rDNA sequences of *Burkholderia pseudomallei* and *Burkholderia mallei* are identical. The 23S rDNA sequences show a single nucleotide difference (C in *B. pseudomallei*, T in *B. mallei*) (Bauernfeind *et al*, 1998). Subsequently, amplification primers were designed that target sequence regions unique in *B. pseudomallei* and *B. mallei* and generate an amplification product that includes the nucleotide position that potentially discriminates the species.

Using these primers a rapid PCR assay was optimised for the detection of *B. pseudomallei*. The duration of the assay was 40 min, a significant reduction from the minimum 2.5 hours required for the conventional method. Table 1 summarises several published PCR methods using a range of primers and cycling conditions. All these assays use conventional thermal cyclers and the duration is at least 2 hours. Currently there is no real time PCR method for the amplification of *B. pseudomallei* described in the literature. The speed of the assay in this report is attributable to a number of factors; faster heating changes due to a combination of increased surface area and the efficient air heating mechanisms of the R.A.P.I.D™ and elimination of the electrophoretic separation of amplified products to both determine success and specificity of assay. The R.A.P.I.D™ procedure also offers a number of useful options for the expansion of the assay. For

example, the existence of multiple channels for different emission wavelengths allows simultaneous amplification and detection of more than one template. This would also allow the inclusion of positive controls in detection assays when unknown samples are being analysed.

Using the amplification conditions optimised for *B. pseudomallei*, the specificity of the assay was assessed using template DNA extracted from several bacterial strains. All of these generated a product when amplified using the universal 16S rRNA primers (Figures 7 and 9). However, the amount of product differed even though the same amount of template DNA was used. Such a difference could be due to the purity of the DNA preparations and/or the copy number of the rDNA operons, the number of which differs in *E. coli* and other bacterial strains (Fegatela et al., 1998). The DNA extracted from *S. sonnei, B. cepacia, S. aureus* and *B. anthracis* strains did not amplify using the conditions optimised for the amplification of *B. pseudomallei* DNA. Melting curve analysis showed that the products formed using template DNA extracted from *C. burnetii, E. coli* and *E. chrysanthemi* exhibited a different melting temperature to the product resulting from *B. pseudomallei* template DNA (Figures 11 and 12). The lower Tm coupled with the broad shape of the melting temperature profile indicates that these products are possibly due to primer-dimer formation. A comparative sequence analysis of the genomes of *C. burnetii, E. coli* and *E. chrysanthemi* with the *B. pseudomallei* primers did not elucidate any regions of greater than 60% homology.

The assay was unable to differentiate the *Y. pestis* product, which was amplified late in the sequence but displayed a similar Tm to the *B. pseudomallei* product. The reasons for cross-reactivity of the *B. pseudomallei* amplification primers with genomic DNA of *Y. pestis* could not be established. A comparative sequence analysis of the *Y. pestis* genome and the *B. pseudomallei* primers did not elucidate any regions within the *Y. pestis* genome of significant homology.

We were unable to test the assay with template DNA from *B. mallei* due to Australian importing constraints. However, the subject of future experiments is to enable discrimination of these species by applying an internal fluorescent probe that targets the potentially diagnostic one-base sequence difference between *B. mallei* and *B. pseudomallei.* The most commonly used fluorescent probes in PCR applications to date are TaqMan hydrolysis probes and hybridisation or FRET (Fluorescence Resonance Energy Transfer) probes (Wittwer, 2001). TaqMan probes carry a fluorochrome and a quencher molecule in close proximity and in this state do not emit a fluorescent signal. When bound to their specific target they are hydrolysed by the 5′-3′ exonuclease activity of the DNA polymerase during extension of the amplification primers. Subsequently, quencher and fluorochrome are separated and a fluorescent signal is detected. TaqMan probes are known to be highly specific but to date no TaqMan probe based PCR assay has been described that allows discrimination of a single base difference. In contrast, hybridisation probes are the tool of choice for detection of SNPs (Single Nucleotide Polymorphisms). Hybridisation probe based PCRs utilise two probes for identification of a specific target. One probe (donor probe) carries fluorescein on the 3′-end. The second (acceptor probe) carries the fluorochrome LightCycler Red 640 or LightCycler Red 705 on the 5′-end and is designed to bind to the target in close proximity to the 3′-end of the donor probe, typically in a distance of one to four nucleotides. During excitation of fluorescein, fluorescence resonance energy is transferred from fluorescein to LightCycler Red and generates a signal

from the acceptor probe. Hybridisation probes enable melting curve analysis of the amplification product and any mismatch between probes and target is identified by the decrease of the melting point (Tm) temperature.

In summary melioidosis, the potentially fatal infection caused by *B. pseudomallei*, is prevalent in regions where Australian troops are likely to be deployed. Currently there is no means of prior immunological protection but treatment if correctly diagnosed in time is effective. Thus there is a need for a rapid detection assay such as the PCR procedure described in this report. The assay is simple and can be used in the field. Even though the assay may not differentiate between the *B. pseudomallei* and *B. mallei* species, the latter is unlikely to be encountered in the Australian environment. In addition, both organisms are susceptible to the same antibacterial agents so treatment for suspected *B. pseudomallei* infections will also target infections caused by *B. mallei*. Similarly, the likelihood of *Y. pestis* in Australia is also minimal. However, in cases when the *B. pseudomallei* PCR is detecting a product late in the assay, a confirmatory PCR for *Y. pestis* should be carried out.

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Appendix A: Results

A.1. Optimisation of *B. pseudomallei* PCR

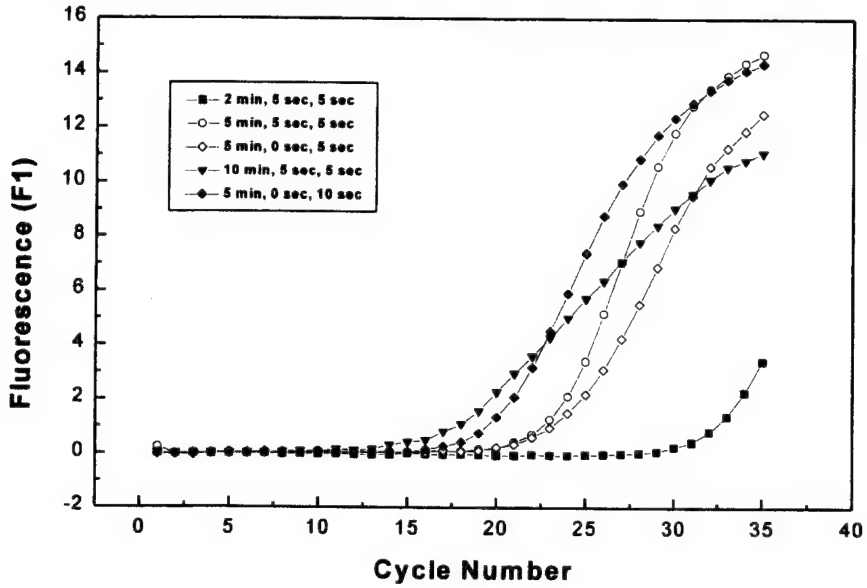

Figure 2: The effect of pre-incubation time on the B. pseudomallei PCR. The times stated in the legend refer to denaturation at 95°C and in the cycling phase denaturation at 95°C and annealing at 55°C, respectively.

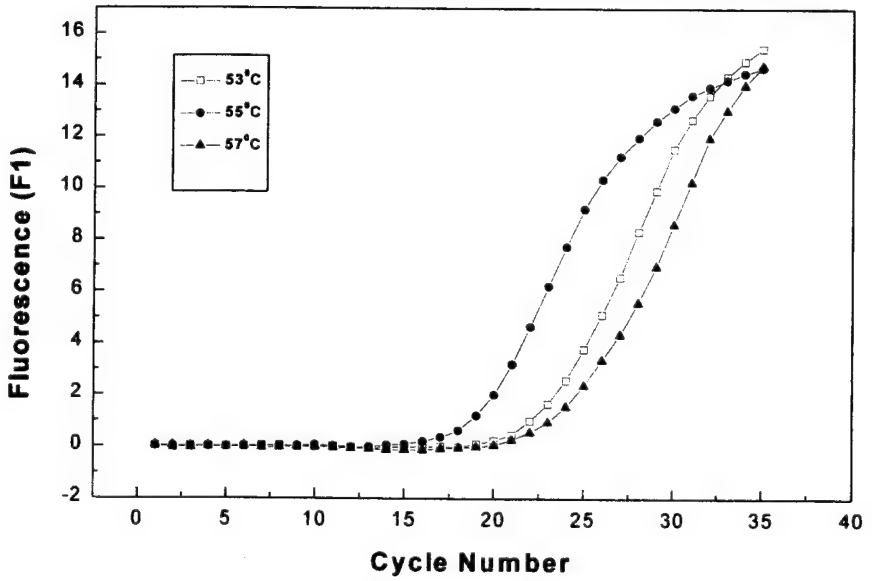

Figure 3: The effect of annealing temperature on the B. pseudomallei PCR.

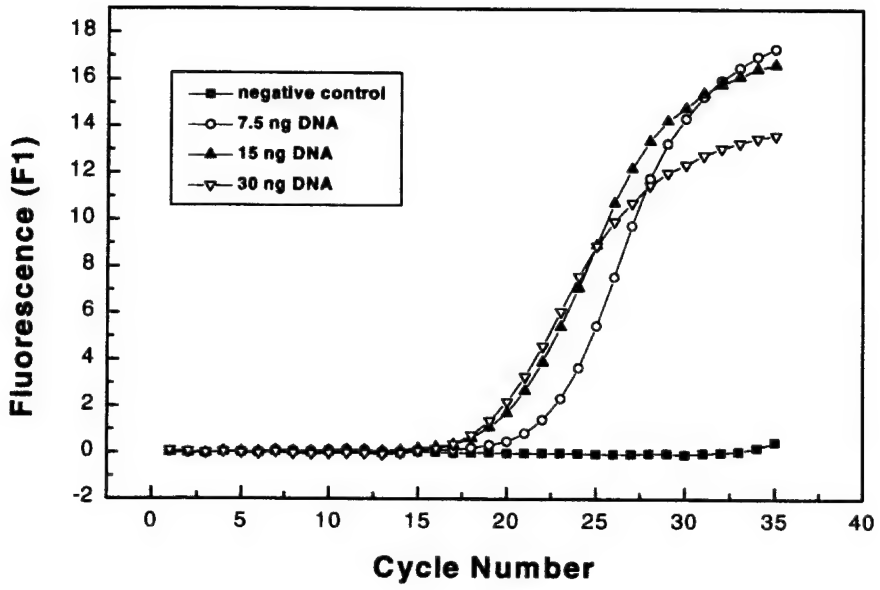

Figure 4: The PCR profile using different amounts of B. pseudomallei genomic DNA template.

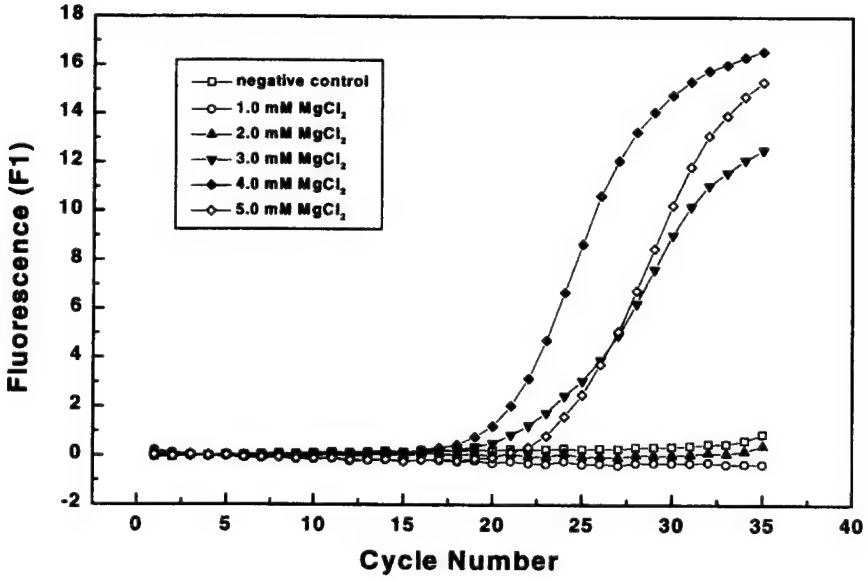

Figure 5: The effect of concentration of $MgCl_2$ on the PCR of the B. pseudomallei fragment.

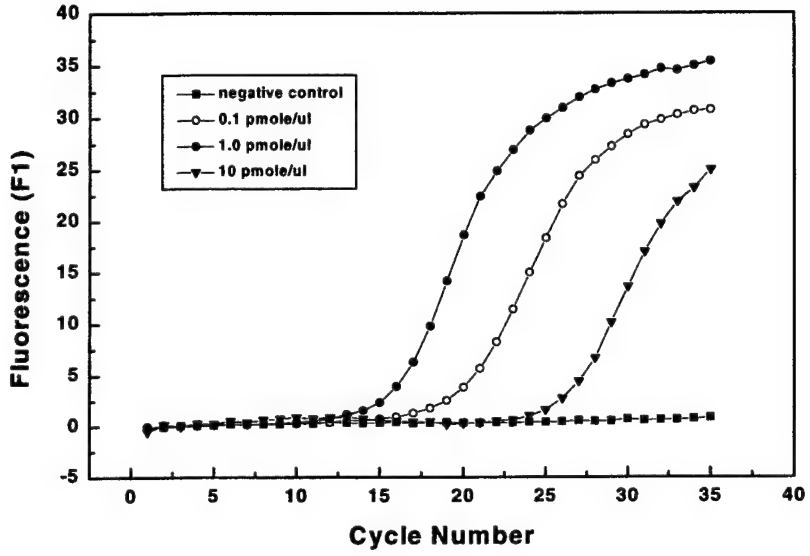

Figure 6: The effect of concentration of the primers BpsF and BpsR on B. pseudomallei PCR.

A.2. Specificity of the *B. pseudomallei* PCR

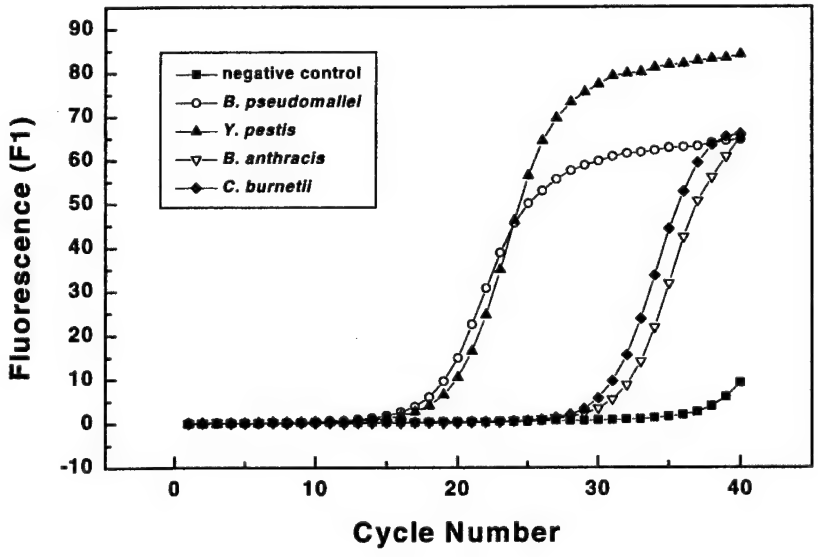

Figure 7: PCR of B. pseudomallei, Y. pestis, B. anthracis and C. burnetii using the universal primers 11-29F and 1492-1512R.

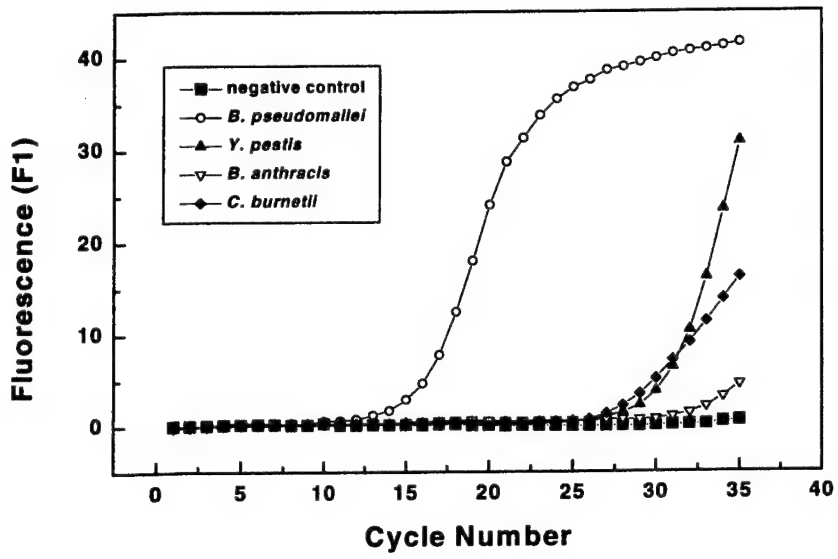

Figure 8: Specificity of the B. pseudomallei PCR. Y. pestis, B. anthracis and C. burnetii genomic DNA were used as templates and reaction carried out using B. pseudomallei PCR cycling conditions.

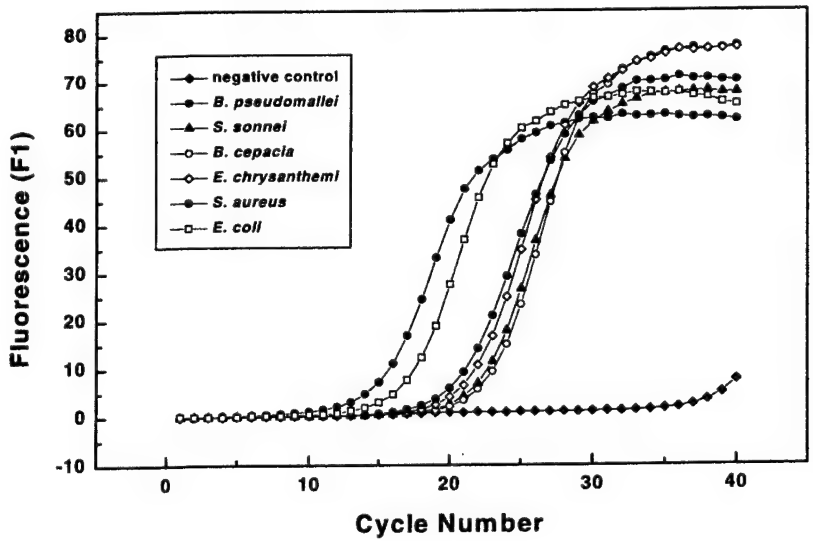

Figure 9: PCR of B. pseudomallei, S. sonnei, B. cepacia, E. chrysanthemi, S. aureus and E. coli using the universal primers 11-29F and 1492-1512R. The reaction was carried out as stated in the methods.

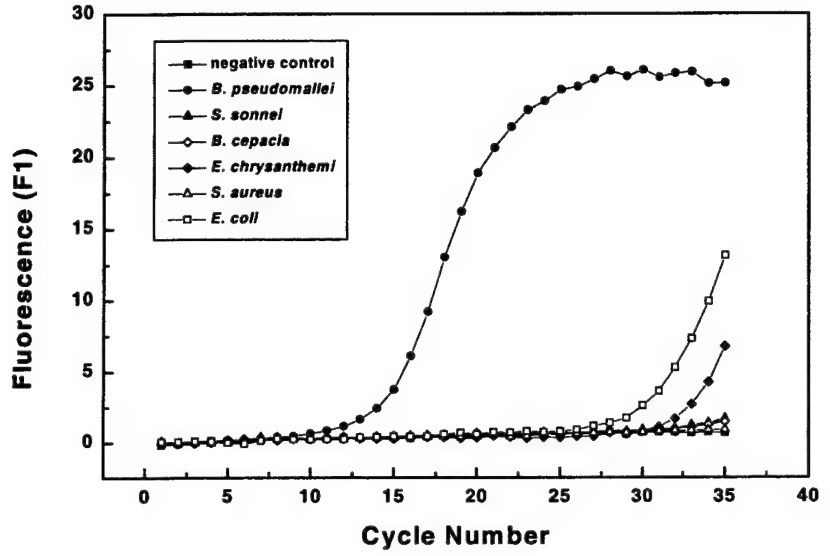

Figure 10: Specificity of the B. pseudomallei PCR. S. sonnei, B. cepacia, E. chrysanthemi, S. aureus and E. coli genomic DNA were used as templates and reaction carried out using B. pseudomallei PCR cycling conditions.

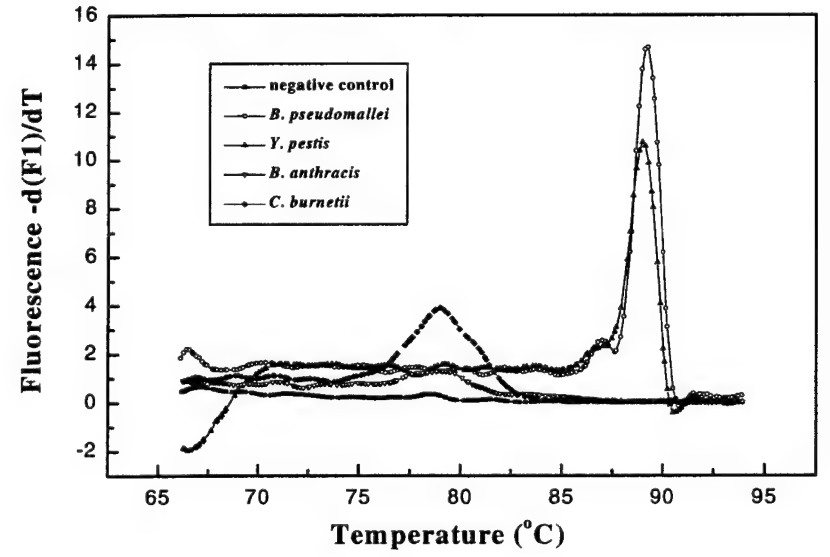

Figure 11: Melting temperature analysis of the PCR products formed using the B. pseudomallei specific primers and cycling conditions

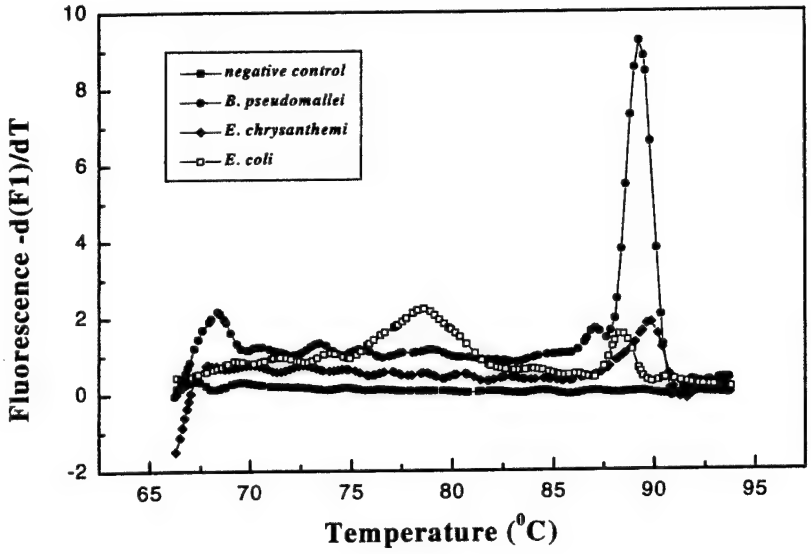

Figure 12: Melting temperature analysis of the PCR product formed using the B. pseudomallei specific primers and cycling conditions

Appendix B: Published PCR methods for detection of *B. pseudomallei*

Table 1: Published PCR methods for detection of B. pseudomallei

Brook et al (1997)

Target sequence	16S rRNA
Primers: PPM3	5'-AATCATTCTGGCTAATACCCG-3'
PPM4	5'-CGGTTCTCTTTCGAGCTCG-3'
Negative with	*B. cepacia, B. gladioli, B. caryophyiil, B.solanacearum, B. picketti, P. syringae, B. andropogonis, Variovorax paradoxus,* Thiobacillus thioparus, Ps. Alcaligenes
Detection limit	10^1 CFU/ml
Assay time	4 hours

Bauernfeind et al (1998)

Target sequence	23SrDNA
Primer 1	5'-CTTTTGGGTCATCCTRGA-3' (*B. viernamiensis, B.mallei,* B..*pseudomallei*)
Primer 2	5'-TCCTACCATGCGAGACT-3' (*B. mallei, B. pseudomallei*)
Primer 1	5'-AAACCGACACAGGTGG-3' (all *Burkholderia* spp.)
Primer 2	5'-CACCGAAACTAGCA-3' (*B. mallei*)
Negative with	*B. cepacia, B. vietnamiensis, B. gladioli, R. pickettii, R. eutropha*
Assay time	2 hours

Lew and Desmarchelier (1994)

Target sequence	23S rDNA
Primers: PPMB2	5'-CCTGCGCGGAACATGTAACGGGGCT-3'
PPM2	5'-CTCTCCTACCATCGAGAC-3'
Negative with	*P. aeruginosa, P. caryophylii, P. gladioli, P. pickettii, P.solanacearum, P. marginalis, P. paucimobilis, P. putida, P. stutzeri*
Positive with	*P. cepacia,*
Detection limit	1.5×10^3 CFU/ml sputum
Assay time	5 hours

Dharakul et al (1996)

Nested PCR:	
Target sequence	16S rRNA
Outer primers:U33	AAGTCGAACGGCAGCACGG
OL731	TTTGCTCCCCACGCTTTCG
Inner primers:BS3L	ACGGGCTTCGGCTGGTG
BS4R	CACTCCGGGTATTAGCCAG
Negative with	*P. putida, P.aeruginosa, P. fluorescenes, E. coli, A. anitratus, K.pneumoniae, E. aerogenes, Proteus* sp., *A. hydrophila, S.marcescens, H. influenzae, S. aureus,* Group A and Group B streptococci, Group D enterococci
Positive with	*B. cepacia* and *S. maltophilia* gave a product in first round
Detection limit	100 CFU/ml
Assay time	1 day

Sura et al (1997)

PCR hybridization:	
Target sequence	not specified
Primers:	5'-ATGCAGATAGCCAACTGCTA-3'
	5'-CGATGAGCTAGGGAATATTGG-3'
Probe:	5'-GTGTGTCTGAAAGTCG-3'
Negative with	*B. mallei, B. cepacia, P. aeruginosa, P. alcaligenes, P.fluorescens, P. pickettii, P. pseudoalcaligenes, P. putida, P. stutzeri, Citrobacter* species, *Enterobacter* species, *E.coli, Klebsiella* species, *M. morganii, P. merabilis, Serratia* species, *C.acidovorans, H. alvei, H. influenzae, S. maltophilia*
Detection limit	35 CFU/ml
Assay time	2 days

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Susan Shahin and Matthias Dorsch

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Susan Shahin and Matthias Dorsch

5. CORPORATE AUTHOR

Platform Sciences Laboratory
506 Lorimer St
Fishermans Bend Victoria 3207 Australia

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Melioidosis, rapid PCR, bacterial disease, diagnosis

19. ABSTRACT

Melioidosis is a potentially lethal infection endemic in Northern Australia and Southeast Asia. The causative bacterium, *Burkholderia pseudomallei*, is resistant to many of the commonly used antibacterial agents. Therefore, successful treatment of the disease is dependent on rapid and accurate diagnosis. The current diagnostic techniques are time consuming and lacking in specificity. We have developed a PCR procedure using the R.A.P.I.D™ and real time monitoring of product formation. The reaction is significantly faster than conventional PCR and allows the simultaneous evaluation of specificity using melting curve analysis.